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# Enhanced Anticancer Effects of Combined Therapy with Quercetin and Rita (P53 Activator) in Hct116 Colorectal Cancer Cells

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**Abstract**

**Background:** Since there is increasing concern about finding improved treatments for the secondleading cancer, colorectal cancer, worldwide, there is a need for improved treatment strategies such as combination therapies, which are expected to be safe and effective. **Objective:** We investigated the anticancer activity of the combination of the natural product quercetin, a plant flavonoid, and the p53-activating agent RITA (reactivation of p53 and induction of tumor cell apoptosis) in HCT116 human colorectal cancer cells. **Results:** The combination index results of the MTT assay revealed that 50+10 μM (quercetin (Que) and RITA) had synergistic effects on the tested HCT116 cells. Similar results were observed in the colony formation, cell migration, apoptosis assays and pathway enrichment analysis. **Conclusion:** The present study's findings demonstrate the synergistic anticancer activity of the combination of Que-RITA **(**50 μM Que and 10 μM RITA) in HCT116 cancer cells.

**Keywords:** Quercetin-Que, Reactivation of p53 and Induction of Tumor Cell Apoptosis-RITA, Combination Therapy, HCT116, Colorectal Cancer

## Introduction

According to the World Health Organization, colorectal cancer is the 2nd leading cause of cancer death worldwide. In 2020, 1.9 million cases were recorded [6], and approximately 9 lakh people died with CRC [1,2]. Combination therapy involving the combination of two or more therapeutic agents [7] is currently considered one of the most important methods for treating cancer [8].

Many dietary compounds rich in flavonoids have been reported to possess strong anticancer properties [3,4].One of such flavonoids is quercetin. It is widely distributed in the human diet and has antioxidant [14], chemo-preventive and anticancer properties [17]. Que has many anticancer effects, such as controlling cell growth signals, blocking the cell cycle at different phases, enhancing proapoptotic effects, and inhibiting the growth of numerous cancer cell types [5,16]. Furthermore, many in vitro and in vivo studies have demonstrated the ability of Que to inhibit the

growth of breast cancer, colon cancer, prostate cancer, ovarian cancer, and lung cancer cells [6]. The anticancer effects of cisplatin, doxorubicin, and other chemotherapeutic drugs have been reported to be enhanced by cotreatment with Que [7,8].

Approximately 60% of human colon cancers are associated with p53 mutations [9]. Although p53-based therapies, which include TP53-based gene therapy, p53 vaccination, small molecule activation of p53, and inhibitors of MDM2, have gained attention, they have limitations due to the absence of multiple targets for cancer cell control [10,11].

Dietary chemo-preventive compounds have gained significant attention because theycould overcome tumour cell resistance to apoptosis [12]. The present research aimed to assess the anticancer activity of Que (natural) and RITA (synthetic) in HCT116 colon cancer cells, which has not been tested thus far. In addition to serving as a wild-type p53 activator, RITA was proven effective regardless of p53 status [13].

The effects of Que on different cancer pathways (by regulating the MEK/ERK, VEGFR2, MEK/JNK, Notch/AKT/mTOR, and PI3K/AKTsignaling pathways) have been well demonstrated [27]. The second drug in combination, RITA, is a p53-MDM2 complex inhibitor that induced DNA damage signalling and enhanced the antiproliferative response to 5FU and oxaliplatin when tested with CRC cells [10,28]. It activates the proapoptotic p53 targets NOXA, PUMA, and BAX while suppressing the expression of the pro-proliferative factors CyclinB1, CDC2, and CDC25C. Additionally, the synergistic effects of RITA and temozolomide inhibited cell proliferation and promoted apoptosis [28].

Considering the above-mentioned anticancer effects of Que and RITA, we investigated the synergistic anticancer effects of Que and RITA in colorectal cancer cells following p53-based apoptosis mechanisms. Additionally, the expression of several key genes involved in oncogenesis, such as RAS, AKT, and JAK/STAT, was investigated.

## Materials and Methods

HCT116 colorectal cancer cells (p53 wild type, ATCC-CCL-247) were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Sigma)

Que (117-39-5) was purchased from Sigma, and RITA (213261-59-7) was purchased from Merck Millipore. Que and RITA stock solutions (1 millimolar) were prepared and stored at -20°C until use. All the other chemicals were procured from Sigma (USA).

**Cells treatment:**

HCT116 cells were seeded in 96-well plates (Thermo Fisher, USA) at a density of ten thousand cells per well in 100 μl of culture medium and incubated at 37°C with 5% $CO_2$ in an incubator. The next day, cells were treated with Que, RITA, combination of Que and RITA and incubated for 48 hrs.

**Cell viability assay**

After treatment, cell viability was determined by MTT cell viability assay[25]. Briefly, the cells were incubated with 100 μg of MTT solution (Sigma, USA) for 4 hrs. The formed formazan crystals were dissolved in DMSO, and the absorbance at 570 nm was measured with a microplate reader (Synergy 4-BioTEK, USA) [4]. The IC50 values (half-maximal inhibitory concentrations) were nonlinearly regressed using GraphPad Prism statistical software and fit a sigmoidal dose-response curve (log of the compound concentration versus the normalized response).

The combinatory effect (CI-combination index value) was analysedusingCompusyn software. Based on these results, 50+10 μM Que+RITA was selected for further assays.

**Colony formation assay**

The clonogenic assay was performed as described[26]. The cells were seeded in 6-well culture plates at a density of 100 cells per well and treated with different concentrations of drugs individually (Que: 25 μM, 50 μM and RITA:10 μM, 20 μM) and in combination with Que+RITA (25 +10, 25+20, 50+10 and 50+20) μM for 48 hr. After treatment, the cells were trypsinized and counted, and then one hundred cells were seeded per drug concentration into fresh 6-well plates and kept in the incubator for 14 days. The media was changed every three days. The colonies were fixed with methanol and stained with 0.5% crystal violet dye for 30 min. Colonies containing more than 50 cells were counted using an inverted phase contrast microscope.

**Cell migration assay**

After cells treatment, the drug-containing medium was removed, a scratch was created with a sterile 200 μl pipette tip, the plates were washed twice with PBS to remove floating cells, and the plates were incubated. Cell migration was monitored by capturing images at 0 and 48 hrs. An inverted phase microscope (Olympus, CKX41) was used. The scratch area was measured relative to the total cell-covered area with Wimasis image analysis software [14].

**Apoptosis assays**

**DAPI staining**

After treatment,cells were fixed with 3.7% formaldehyde in PBS (10 min, room temperature)and then washed three times for 10 minutes each in PBS. The cells were incubated for 5 min in 0.1% Triton X-100 (Sigma) for permeabilization. After being washed and mounted, the cells were incubated for 10 minutes in the dark at room

temperature with DAPI (stock =1 mg/ml in distilled water diluted 1:1000 in PBS before use). The slides were screened, and the number of cells showing nuclear changes characteristic of apoptosis were expressed as the percentage of apoptotic cells [15].

**Effects of drug combinations on the cell cycle: Flow cytometry**

After treatment, the collected cells were washed with cold PBS, fixed in 70% ethanol at 4°C, and stored for 24--48 hrs. at −20°C. Before analysis, the cells were washed with cold PBS, suspended in DNAse-free RNAse A (420 µg/ml, Biomedicals, LLC, France) in PBS at 37°C for 20 minutes, and stained with propidium iodide (PI) at a concentration of 1 µg/ml. The cell cycle phase distribution of drug-treated HCT116 cells and untreated cells was measured using BD FACS Analyser 561 (FortessaAnalyser,Becton, Dickinson, and Company (BD), USA).

**Estimation of caspase 3 and 9 activity**

The activities of caspase-3 and caspase-9 were estimated using colorimetric assay kits obtained from Enzo Life Sciences (USA). After treatment, the cells were trypsinized, and the pellet was washed with PBS. After treatment, cell lysates were prepared, and caspase-3 and caspase-9 levels were estimated according to the manufacturer's instructions.

**Western blotting**

The expression of various key apoptotic proteins involved in apoptosis (proapoptotic, antiapoptotic, p53 and p53 inhibitors) and PI3K and mTOR pathway proteins (AKT-Thr308 and AKT-Ser473) was estimated by Western blotting.

**Pathway enrichment analysis**

The NanoString nCounter gene expression assay was conducted by a contract research organization (Theracues Pvt Ltd, Bangalore, India) via validated commercial methods on a pay-by-service basis. Briefly, RNA was extracted from HCT116 cell samples using a commercial FFPE nucleic acid isolation kit (Roche Molecular Diagnostics).

The differentially expressed (DE) genes in the HCT116 cell samples (control, Que, RITA, and Que+RITA) were identified and analysed by Resolver 4.0. The significant genes were subjected to principal component analysis (PCA), and a heatmap was generated via the ClustVis webserver. The assay involved probing for an interesting panel (nCounter Hs Pan-Cancer Pathway Panel) of genes in the samples.

**Statistical Analysis**

The experiments were carried out in triplicate. GraphPad Prism Software version 8.0 (San Diego, USA) was used to evaluate the results, recorded as the mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by Dunnett's multiple comparison test wasdetermined (level of significance $P < 0.05$).

## Results

### Cell viability assay

Compared with the control, both drugs effectively inhibited HCT116 cell proliferation and a significant reduction in cell viability was observed in a dose-dependent manner with 25–250μM Que (Fig. 1B) and 40 μM RITA (Fig. 1C). The calculated IC50 values of Que and RITA were 48.55 and 20.74 μM, respectively.

The effect of the drug combination on HCT116 cells was evaluated by MTT cell viability assay. The cells were treated with four combinations of Que+RITA (25+10, 25+20, 50+10 or 50+20) μM and individual concentrations of Que (25 or 50) μM or RITA (10 or 20) μM for 48 hr. The cell viability was estimated by MTT assay, and drug combination effects were evaluated byCompusyn analysis (Table 1). When the cells were observed under a microscope (Fig. 1A), maximum cell death was observed after 48 hrs. incubation.

**Fig 1.** Cell viability assay results

**Table 1.** Drug combination effects – MTT cell viability assay, Combination effect analysis – Compusyn assay

For all the tested drug combinations, Que+RITA (50+10 μM) had lower CI values, indicating greater synergistic drug combination effects at the lowest possible concentrations of RITA. On the basis of the results of this test, 50+10 μM Que+RITA was used for further anticancer assays.

### Colony formation assay:

A colony formation assay was conducted with 25 and 50 μM Que and 10 and 20 μM RITA in cultured HCT116 cells individually or in combination with Que+RITA. A dose-dependent effect on the inhibition of colony formation was observed for the individual drug treatments, and greater effects on the inhibition of colony formation were observed for the combination treatments (Fig 2). A statistically significant reduction in colony formation ($p < 0.05$) was observed in both the individual and combination drug treatment groups compared with the control group.

**Fig 2**. Effect of the combination of Que and RITA on colony formation inhibition in HCT116 cells.

### Cell migration assay:

The migration of HCT116 cells treated with Que, RITA, or a combination of Que and RITA was calculated at 0 hr. and 48 hrs. using the following formula:

% Cell migration = scratch area at 0 hrs. - Scratch area at 48 hrs./Scratch area at 0 hrs. ×100.

In the control group, 100% of the cells migrated into the scratch within 48 h, resulting in complete closure of the scratch, and the extent of cell migration in the drug-treated groups was calculated at time intervals (Fig. 3). The observed percentages of cell migration were 42.86 and 40.00% for the Que (50 μM) and RITA

(10 μM) individual treatments, respectively, and 22.22% for the Que+RITA (50+10 μM) drug combination treatment.

**Fig 3.** Combined effect of Que and RITA on HCT116 cell migration in vitro

**Apoptosis assays:**

**DAPI staining:**

Apoptotic cells were observed for morphological changes in the nucleus after treatment with Que, RITA,and combination. The results are shown in Fig. 4A, 4B. Thepercentage of apoptotic cells after treatment with Que (50 μM), RITA (10 μM), and their combination were 11.3%, 10%, and 29.7%, respectively. A statistically significant increase in the percentage of apoptotic cells ($p < 0.05$) was observed in the combination drug treatment group compared with the untreated control group.

**Cell cycle analysis by flow cytometry:**

For the cell cycle analysis, the percentage distribution of cells in the G1, S, and G2 phases along with the results of the quantitative analysis of each phase of the treated cells were compared with the untreated control cells. A significant increase in the proportion of cells in the sub-G1 phase was observed after treatment with the drug combination, indicating increased apoptosis. The results of the flow cytometric analysis are shown in Fig. 4F, 4G, 4H, and 4I.

**Treatment with Que and RITA increased caspase activity:**

Treatment with Que (50 μM) + RITA (10 μM) and their combinations (50+10 μM) increased caspase activity (caspase-3 and 9) in HCT116 cells, resulting in 2.2-, 2.1-, and 3.7-fold increases in caspase-3 activity and 2.0-, 1.8- and 3.4-fold increases in caspase-9 activity, respectively (Fig. 4C, 4D).

**Western blot analysis:**

Treatment with the drugs Que, RITA, and their combination resulted in 0.99-, 0.59-, and 0.51-folddecrease in the Bcl-2 (antiapoptotic) protein; 1.27-, 1.30-, and 1.36-folds increase in the BAX (proapoptotic) protein; and 1.07-, 1.51-, and 1.59-foldsincrease in p53 activity, respectively, as illustrated in Fig. 4E.

Drug combination effects on the PI3K and mTOR pathways in HCT116 cells:

Treatment of HCT116 cells with Que (50 μM), RITA (10 μM) and in combination resulted in 0.74-, 0.47-, and 0.41-folds decrease in AKT-Thr308 protein expression and 0.54-, 0.47- and 0.26-folds decrease in AKT-Ser473 activity, respectively, illustrated in Fig. 4E.

**Fig 4.** Apoptosis Assays-Results

**Pathway enrichment analysis:**

In this assay, we observed that Quercetin+RITAcombinationresulted in many differentially expressed genes compared with the other treatment conditions (control, Que, and RITA) in a panel containing 730 target endogenous genes along with 40 housekeeping genes (NS_CANCERPATH_C2535.rlf, nSolverTM analysis

software (version 4.0; NanoString Technologies), as illustrated in the distribution of DEGs in Fig. 5A and heatmaps in Fig. 5B&C.

**Fig 5.** Pathway enrichment analysis results

## Discussion

Given the potential anticancer effects of herbal combinations, often more effective and safer than synthetic agents, this study explored the synergistic anticancer activity of the plant flavonoid Que and the potent p53 activator RITA in HCT116 cell lines.

Que induced proapoptotic effects by regulating the p53 gene and BCL-2 protein both in vitro and in vivo. The effect of Que on p53-mediated apoptosis in various cancer cell lines has been investigated[17].

In the flow cytometry analysis, the percentages of apoptotic cells in the sub-G1 phase were 2.38 (control), 11.55 (10 μM RITA), 11.27 (50 μM Que), and 13.76 (50 μM Que and 10 μM RITA). The results of the DAPI staining were consistent with the flow cytometry results (Fig. 4). Upregulation of p53 and proapoptotic proteins and downregulation of antiapoptotic proteins were observed via gene expression analysis and western blotting. The results of the caspase activity assay were correlating withother apoptotic assays.

In the context of synergistic effects, Que hasbeen shown to have synergistic effects with doxorubicin [18], aconitine [19], cisplatin, and 5-fluorouracil [20] in various cancer cells [21]. In a previous study, Que effectively reversed paclitaxel resistance by inhibiting Akt and ERK phosphorylation and altering the membrane potential in A549/Taxol cells [22].

Reactivating p53 and inducing tumor cell apoptosis (RITA) has been shown to increase p53 function [23] and induce p53-dependent apoptosis in cancer cells by promoting the degradation of MDM2, thereby activating the p53 downstream program [24].

In the present study, the gene expression analysis results correlated with the results of the antiproliferative and apoptotic assays, in which many of the genes involved in or that induce apoptosis either directly or indirectly were upregulated (BAX, Tp53, CASP3, CASP9, and mTOR). The genes responsible for downregulating apoptotic pathway genes (BCL2, RAS, JAK, STAT, PI3K, NFKB and NOTCH) were expressed at low levels(Fig. 5).

The results of this study indicate that the combination treatment of Que and RITA has effective antiproliferative effects on HCT116 cells because of its robust synergistic effect.

## Conclusion

The selected drug combinations inhibited the p53 apoptotic pathway, including the RAS and AKT pathways. These findings serve as a basis for conducting additional anticancer assays targeting combination therapy for colorectal cancer.

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## Author contribution statement

**T. Yasodha Lakshmi:** Data curation, Resources, Writing – original draft.
**P.Chandra Sekhar:** Conceptualization, project administration, software, supervision, formal analysis, and investigation writing – review & editing.
**Subramani Rammurthy:** Supervision,review & editing
**Shama Prasada Kabekkodu:** Methodology, review& editing.

## Declaration of competing interest

All the authors contributed significantly to this work and approved the final version.
Yasodha Lakshmi Tadakalurudeclares that she has no conflict of interest
Chandra Sekhar Pasuladeclares that he has no conflict of interest
Rama Murthy Subramani declares that he has no conflict of interest
Shama Prasada Kabekkodu declares that he has no conflict of interest

**Ethical approval**: 'This article does not contain any studies with human participants or animals performed by any of the authors.

## Data availabilitystatement

**"**The data sets generated during the current study are available from the corresponding author on reasonable request.''

## Abbreviations:

AKT-Ak strain transformation
BAD-Bcl-2 antagonist of cell death
BAX-Bcl-2-associated protein x
BCL-B-cell leukemia
CASP-Caspase
CDC–cell division cycle
CI-combination index
CRC-Colorectal cancer

DAPI-4′,6-diamidino-2-phenylindole
DE-differentially expressed
DMSO-Dimethyl sulfoxide
DNA- Deoxyribonucleic acid (DNA)
ERK-Extracellular signal-regulated kinase
FFPE-Formalin-Fixed Paraffin-embedded
G1-growth 1 phase
HCT-human colorectal carcinoma reporter gene cell lines
HS -High sensitivity
IC-Inhibitory concentration
JAK-Janus kinase
JNK-Jun N-terminal kinase
mA-milliampere
MAPK-mitogen-activated protein kinase
MCL-myeloid cell leukemia sequence
MDM2-Mouse double minute 2 homologue
MEK-mitogen-activated extracellular signal-regulated kinase
mTOR-mammalian target of rapamycin
MTT-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
μl-Micro liter
μM-Micromolar
NFKB-Nuclear factor kappa B
NOTCH-Neurogenic locus notch homologue protein
NOXA/PMAIP1-phorbol-12-myristate-13-acetate-induced protein 1
PARP-Poly (ADP–ribose) polymerase
PBS-Phosphate-buffered saline
PCA-Principal component analysis
PI-Propidium iodide
PI3K-phosphatidylinositol 3-kinase
PUMA-p53-upregulated modulator of apoptosis
Que-Quercetin
Ras/RAS-Rat sarcoma
RIPA-Radioimmunoprecipitation assay buffer
RITA reactivates p53 and inducestumor apoptosis
RNA- ribonucleic acid
rpm-Revolutions per minute
SDS–PAGE–Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
STAT-Signal transducer and activator of transcription
TBST-Tris-buffered saline with 0.1% Tween® 20 detergent
Tp53-tumor protein p53 gene

TUNEL-terminal deoxynucleotidyl transferase dUTP nick end labelling
VEGFR2-Vascular endothelial growth factor receptor 2
V-Volts

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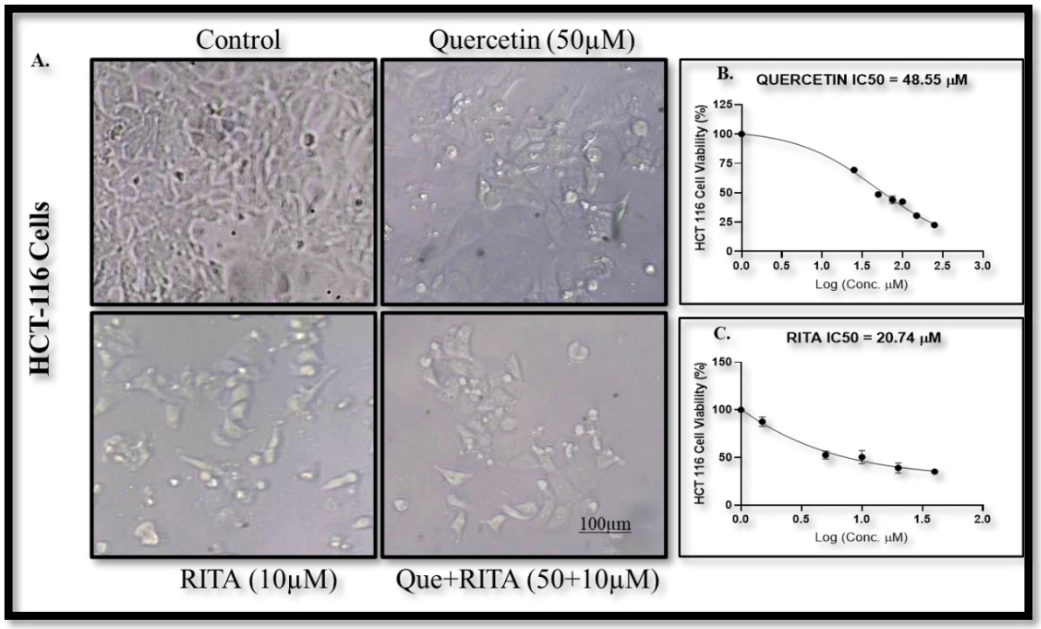
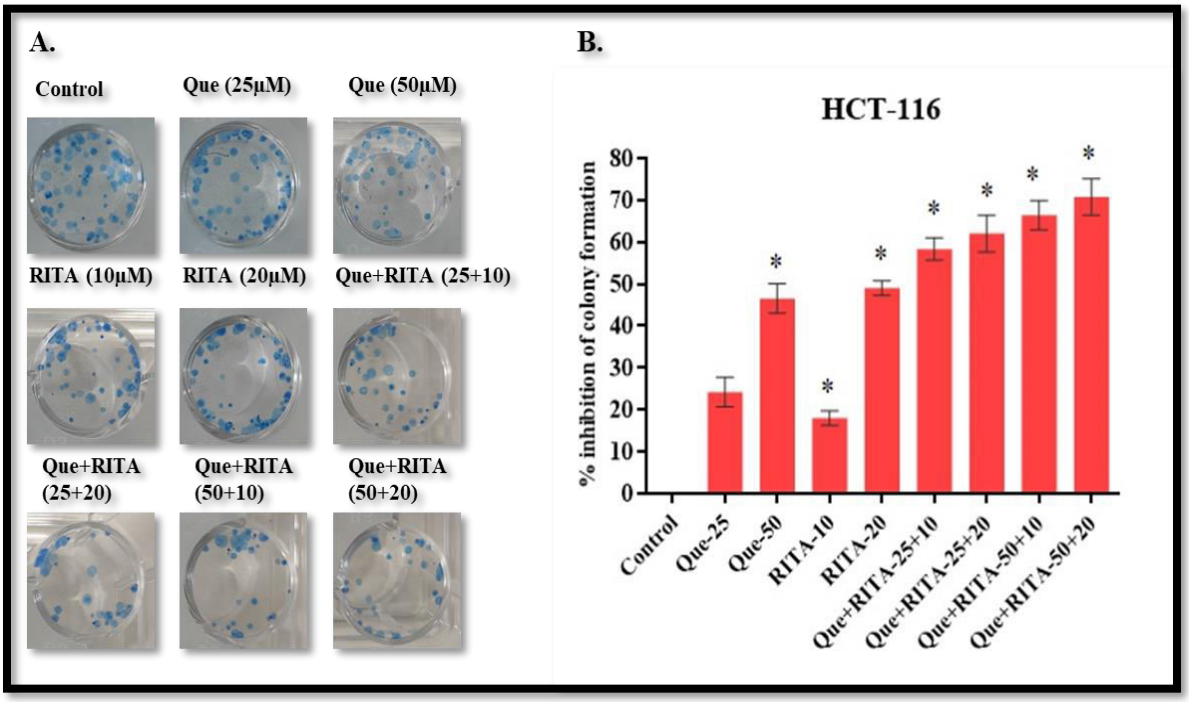
**List of Figures**

**Fig 1.** Cell viability assay results



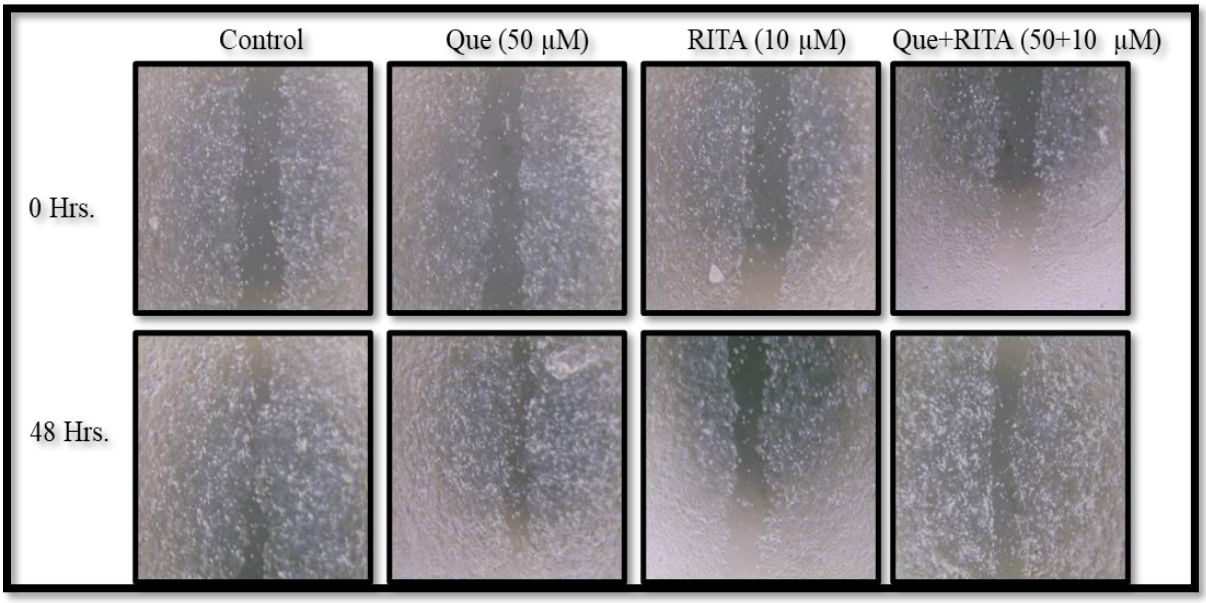

**A.** Microscopy images of cells after treatment with 50 μM Que, 10 μM RITA or their combination for 48 hr. (Scale bar, 100 μm). **B.** Dose-dependent curve for the IC50 calculation of Que. **C.** Dose-dependent curve for the IC50 calculation of RITA. The data are expressed as the means ± SDs (n=3). Compared with the control, Que and RITA had anti-proliferative effects that were directly proportional to the dose in the tested HCT116 cells. DMSO (0.1%) was used as a control.

**Fig 2.** Effect of the combination of Que and RITA on colony formation inhibition in HCT116 cells.



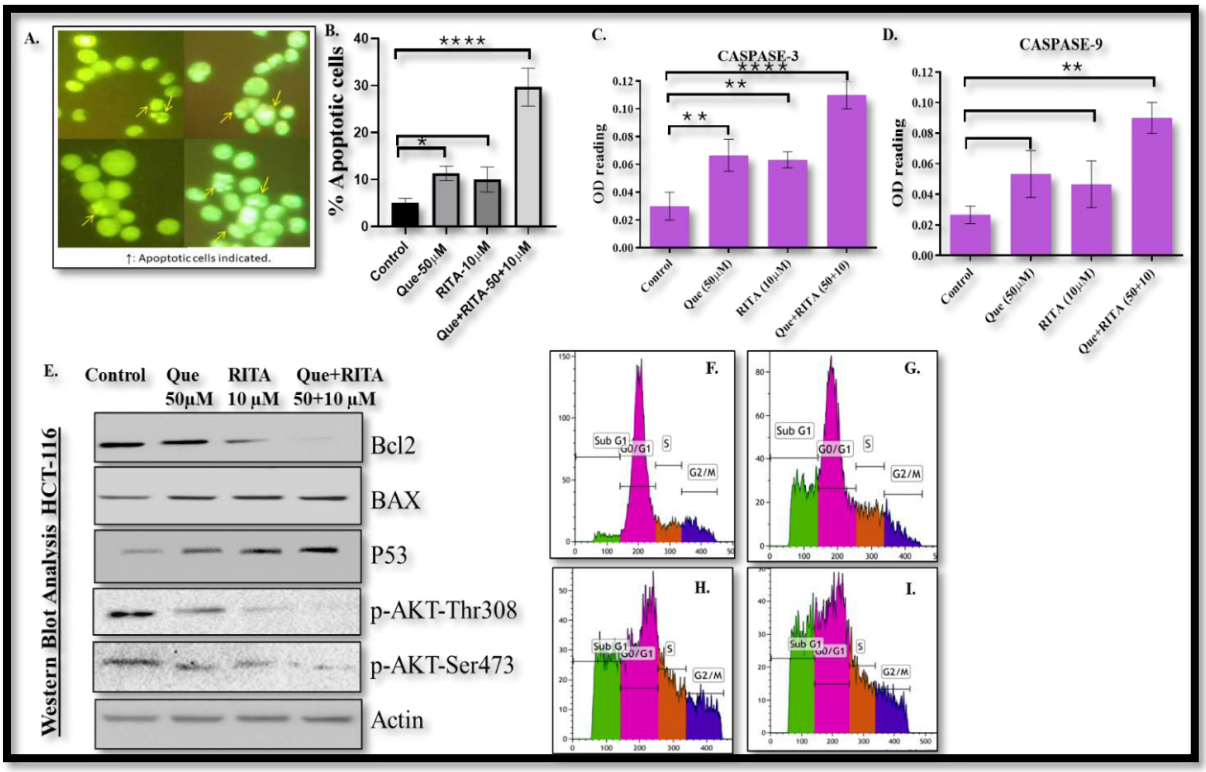

**A.**-Stained colonies in culture plates corresponding to the drug combinations. **B.** Percent inhibition of colony formation by different drug combinations compared with the control. The data are expressed as the means ± SDs (n=3). *$p < 0.05$.

**Fig 3.** Combined effect of Que and RITA on HCT116 cell migration in vitro



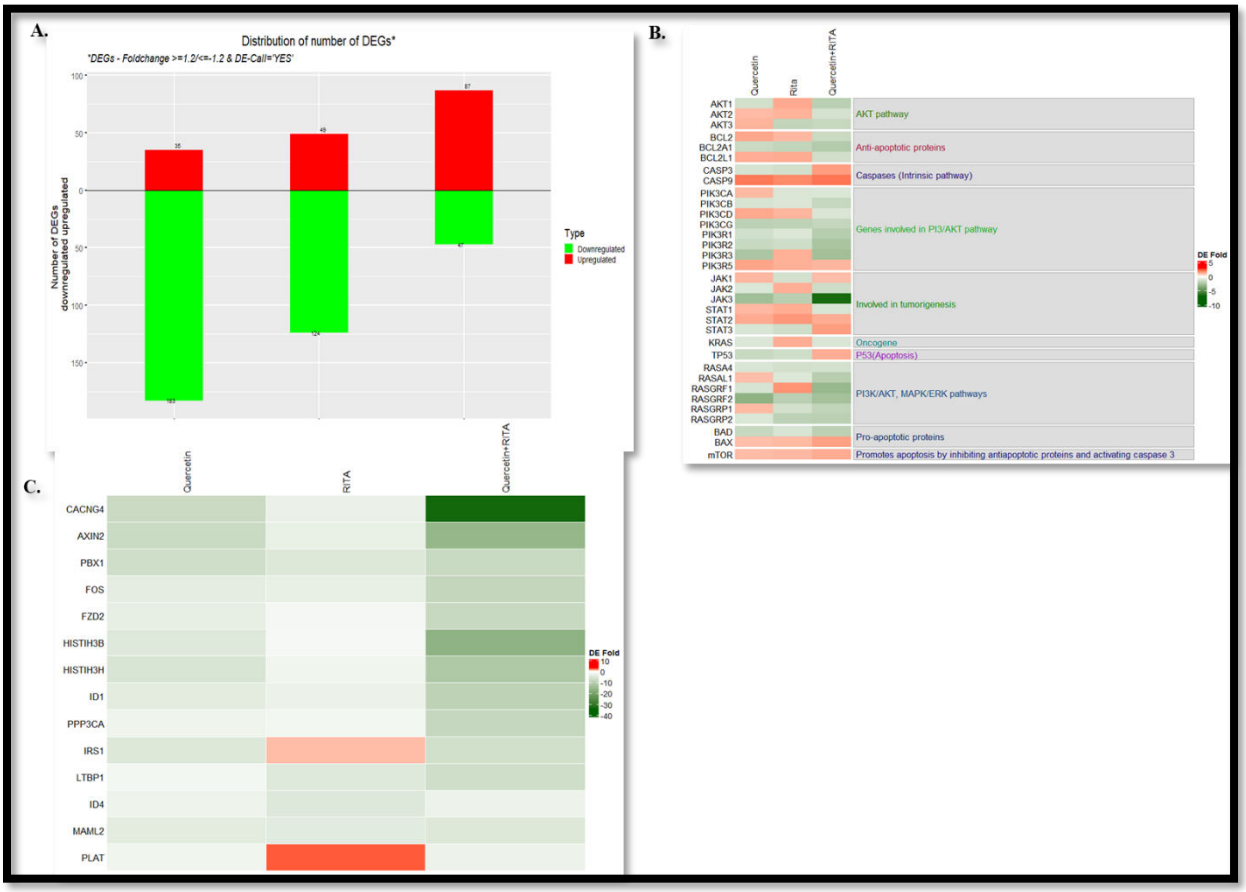

Images were captured to monitor cell migration at 0 and 48 hrs. and analysed by Wimasis image analysis software.

**Fig. 4.** Apoptosis Assays--Results



**A.** Fluorescence microscopy images of cells after DAPI staining (cell shrinkage, fragmentation, and nuclear condensation, as shown in the figure, were quantified by counting the number of apoptotic cells). **B.**Effects of the combination of Que and RITA (50 μM +10 μM) on the apoptosis of HCT116 cells. The data are expressed as the means ± SDs (n=3). *$p < 0.05$, ****$p < 0.0001$. **C, D.** Drug combination effects on caspase-3 and caspase-9 activity. The OD values of treated cells were compared with those of control cells to evaluate the fold increase in caspase activity. In HCT116 cells, the increase in caspase-3 activity was 3.7× for the combination treatment, and the increase in caspase-9 activity was 3.4× for the combination treatment. The data are expressed as the means ± SDs (n=3). **$p < 0.01$, ****$p < 0.0001$. **E.** Western blot image of the effects of the drug combination on apoptosis (proapoptotic effects: p53↑ and BAX↑; antiapoptotic effects: Bcl2↓) and the PI3K and mTOR pathways (p-AKT-Thr308↓ and p-AKT-Ser473↓, respectively) in HCT116 cells. ↑-increase, ↓-decrease, protein quantification by ImageJ software, where drug-treated values are relative to those of the untreated control, which was considered 1. β-Actin was used as a loading control.**F.** Cell distribution in the control group. The total percentage of cells in the subG1 phase was 4.0. **G.** The total percentage of cells treated with 10 μM RITA in the subG1 phase was 24.0. **H.** The total percentage of cells treated with 50 μM Que in the subG1 phase was 25.0. **I.** The total percentage of cells treated with 50 μM Que and 10 μM RITA in the subG1 phase was 27.0. The cell cycle of HCT116 cells arrested in the sub-G1 phase after 48 hrs. of treatment.

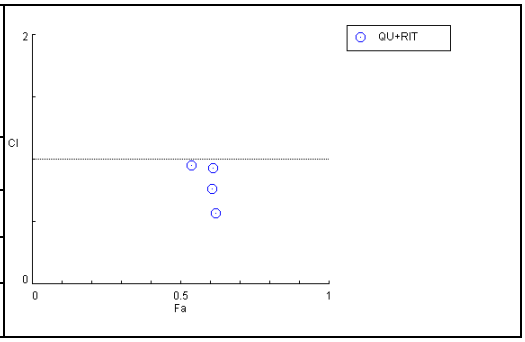
**Fig 5.** Pathway enrichment analysis results




**A.** Data Bar1- Que+RITA vs. Control (No. of genes:183↓, 35↑), Data Bar2- Que vs. Control (No. of genes:124↓, 49↑), Data Bar3- RITA vs. Control (No. of genes:47↓, 87↑), ↓-downregulated, green in color, ↑-upregulated, red. **B.** Heatmap showing the 33 DE genes related to the apoptosis pathway. DE Fold – Treated group/Control group; the color indicates the following: orange, gene upregulation; green, gene downregulation. DE call: Fold change> 1.2 (upregulated) or Fold change< 1.2 (downregulated). **C.** Heatmaps showing the DE (differentially expressed) genes related to cancer cell proliferation and the angiogenesis pathway.

**List of Tables**

**Table 1.** Drug combination effects – MTT cell viability assay, Combination effect analysis – Compusyn assay

| Combination QUE+RITA (μM) | CI Value /Drug combination Effect |  |
|---|---|---|
| 25+10 | 0.96/Additive effect | |
| 25+20 | 0.77/Synergistic effect | |
| 50+10 | 0.57/Synergistic effect | |
| 50+20 | 0.93/Additive effect | |